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In vivo genome editing as a potential treatment strategy for inherited retinal dystrophies

Mert Yanik [a, 1], Brigitte Müller [a, 1], Fei Song [a, 1], Jacqueline Gall [a, 1], Franziska Wagner [a, 1], Wolfgang Wende [b, 1], Birgit Lorenz [a, 1], Knut Stieger [a, *, 1]

[a] Department of Ophthalmology, Faculty of Medicine, Justus-Liebig-University Giessen, Germany
[b] Institute of Biochemistry, Faculty of Biology and Chemistry, Justus-Liebig-University Giessen, Germany

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ABSTRACT

In vivo genome editing represents an emerging field in the treatment of monogenic disorders, as it may constitute a solution to the current hurdles in classic gene addition therapy, which are the low levels and limited duration of transgene expression. Following the introduction of a double strand break (DSB) at the mutational site by highly specific endonucleases, such as TALENs (transcription activator like effector nucleases) or RNA based nucleases (clustered regulatory interspaced short palindromic repeats - CRISPR-Cas), the cell's own DNA repair machinery restores integrity to the DNA strand and corrects the mutant sequence, thus allowing the cell to produce protein levels as needed. The DNA repair happens either through the error prone non-homologous end-joining (NHEJ) pathway or with high fidelity through homology directed repair (HDR) in the presence of a DNA donor template. A third pathway called microhomology mediated endjoining (MMEJ) has been recently discovered. In this review, the authors focus on the different DNA repair mechanisms, the current state of the art tools for genome editing and the particularities of the retina and photoreceptors with regard to *in vivo* therapeutic approaches. Finally, current attempts in the field of retinal *in vivo* genome editing are discussed and future directions of research identified.

Contents

* Corresponding author. Department of Ophthalmology, Faculty of Medicine, Justus-Liebig-University Giessen, Friedrichstr. 18, 35392 Giessen, Germany.
E-mail address: knut.stieger@uniklinikum-giessen.de (K. Stieger).

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1. Introduction

Inherited retinal dystrophies are a group of disorders with a prevalence of 1 in 3–4000 people (Hartong et al., 2006). Owing to the complexity of the visual system, several hundred proteins are more or less uniquely expressed in the retina, and mutations in over 200 genes have been associated with retinal dystrophies (https://sph.uth.edu/retnet/) (Berger et al., 2010). Specific advantages of the eye as target tissue have positioned this organ at the forefront of gene therapeutic development over the last 20 years, be it gene addition therapy by use of viral vectors for the transfer of correct cDNA copies of mutated genes, or gene silencing using RNA interference to knock down proteins with dominant negative effects or toxic gain of function activities. In addition, exon skipping approaches to neutralize mutations in single exons or the expression of neuroprotective proteins to keep photoreceptor cells alive as nonspecific gene therapy approach have been tested (for review of recent advancements (see Petit et al., 2016).

The very active field of retinal gene addition therapy has advanced the most with several clinical trials ongoing for *RPE65* and *MERTK* deficiency (associated with mutations in the *RPE65* or *MERTK* gene), choroideremia associated with mutations in *REP1*, Stargardt macular dystrophy associated with mutations in *ABCA4*, juvenile retinoschisis associated with mutations in *RS*1, and Usher syndrome 1B associated with mutations in *MYO7A* (Boye et al., 2013). For one entity, *RPE65* deficiency, a treatment based on recombinant adeno-associated virus (AAV) vectors expressing the human *RPE65* cDNA under the control of a viral promoter is about to reach approval by the FDA and EMA, emphasizing the relative success with this treatment (Schimmer and Breazzano, 2015).

However, all these approaches aim at neutralizing the symptomatic problem, that is the lack of a given protein production in case of a nonsense mutation or the production of a non-functional protein in case of a missense mutation, but leave the cause unaddressed, i.e. the disease causing mutation. Furthermore, some autosomal dominant diseases are caused by gain-of-function mutations that are not addressed by gene addition therapy. What's more, artificial expression systems employing viral promoters and polyadenylation signals without intracellular sensor very likely do not lead to physiological levels of protein production, which may hinder the cell to reach the optimal equilibrium of protein levels. Not to mention the effect proteins expressed from genes with missense mutations may have on the production rate or function of transgenic proteins.

Targeted genome editing may solve these issues by correcting the disease causing mutation within the genome in order to restore the “wild type” DNA sequence of a given gene, enabling the cell to produce what is needed to have optimal phenotypic outcome (Cox et al., 2015). Genome editing is based on the cells' own capacity to repair DNA double strand breaks (DSB), which are the most dangerous form of DNA damage that can occur to a cell. Frequently happening during mitosis in case of a stalked replication fork or ionic radiation, DSBs are repaired either by sticking the DNA ends together by a mechanism called nonhomologous end-joining (NHEJ), or by use of the sister chromatid as template DNA via homology directed repair (HDR) (Jasin and Haber, 2016). A third way to repair DSB represents a mechanism called micro-homology mediated endjoining (MMEJ). While DSBs occur sporadically and throughout the entire genome (Lindahl, 1993), the frequency of DSBs can be dramatically increased and targeted to a defined locus within the genome by use of sequence specific endonucleases, such as Meganucleases, zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN) or RNA based nucleases (clustered regularly interspaced short palindromic repeats = CRISPR based systems). By use of DNA templates containing homologous DNA sequences at both ends, endonuclease based genome editing is currently used to rapidly generate animal models or insert/remove DNA sequences or genes from hematopoietic cells *ex vivo* in human clinical trials (Osborn et al., 2016).

The idea of using targeted genome editing to repair disease causing mutations is comparatively young, and relies on highly specific endonucleases and the capacity of the cell to repair DSB. Overall, the development of therapeutic strategies leads currently into two directions: (i) *ex vivo* genome editing, and (ii) *in vivo* genome editing (Fig. 1). *Ex vivo* approaches are based on the idea to take a skin biopsy, de-differentiate the cells into induced pluripotent stem cells (iPSCs), correction of the mutation by gene transfer, re-differentiation of selected cells into RPE or photoreceptor cells and re-implantation of these cells into the retina. Here, the cells to be modified by genome editing re-enter cell division and can thus be much more easily treated, screened and selected for successful genome editing.

Alternatively, *in vivo* approaches aim at treating the mutations directly in retinal cells in situ. There is no need for *ex vivo* de-differentiation, re-differentiation and re-implantation. Vehicles for *in vivo* gene transfer exist in form of virus based vectors, such as AAVs. The major drawback here is the post-mitotic state of the cells, which very likely hinders efficient genome editing, and the absence of screening and selection possibilities. It is therefore absolutely necessary to know what we do when modifying the genome in order to avoid unwanted side effects due to the treatment.

Taken together, *in vivo* genome editing in the retina involves several steps that are unique or particularly difficult to establish (summarized in Fig. 2) compared to other organs, making the entire concept highly risky. At the same time, the advantages of such a system for the treatment of many different monogenic disorders make it worth taking a large effort to bring it to reality.

In this review, the authors focus on the different DNA repair

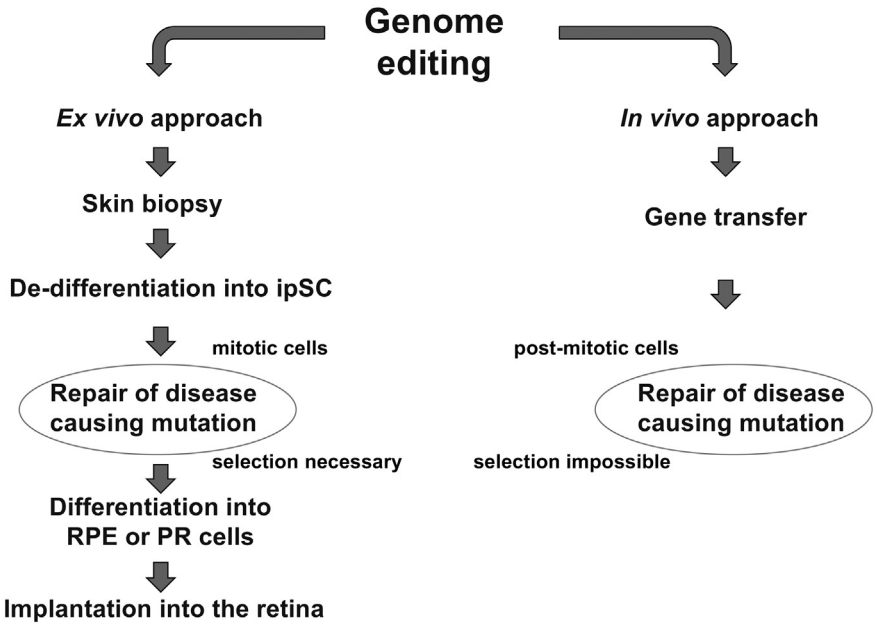

Fig. 1. General Scheme of genome editing in the retina, distinguishing *ex vivo* and *in vivo* approaches.

mechanisms, the current state of the art tools for genome editing and the particularities of the retina and photoreceptors with regard to *in vivo* therapeutic approaches. Finally, current attempts in the field of *in vivo* genome editing in the retina will be discussed and future directions of research identified.

2. The tools

2.1. Endonucleases

Genome editing is advancing at an exceptionally rapid pace, with huge impacts on biotechnology and biomedicine. Trigger for this revolution was the research transition from companies and specialized laboratories using engineered meganucleases and designed zinc finger nucleases as tools to virtually any molecular biology laboratory with the introduction of the TALEN and CRISPR/Cas technology. This groundbreaking development was facilitated by the dissemination of plasmids harboring these new tools to the research community by nonprofit organizations like addgene. All tools initiate the genome editing process usually by the introduction of DSBs or single strand breaks (nick), whose subsequent repair results in the genomic modification. This was first demonstrated in the early-1990s using the highly specific homing endonuclease I-SceI (Puchta et al., 1993; Rouet et al., 1994). These results launched the run for customized nucleases, of which I-SceI, recognizing an 18 bp long sequence, remained the gold standard for cleaving genomic DNA.

2.1.1. Meganucleases

To exploit the highly specific LAGLIDADG homing endonucleases, also called meganucleases, for gene targeting, their DNA recognition properties (14–40 bp) had to be redesigned to cleave any given sequence (Silva et al., 2011; Stoddard, 2014). Combinations of computational redesign, *in vivo* selection, artificial dimers and high-throughput screening procedures were used to generate meganucleases with tailored specificities (Ashworth et al., 2006; Smith et al., 2006; Pingoud and Wende, 2011; Takeuchi et al., 2015). Based on I-CreI as a scaffold, naturally binding a palindromic sequence of 22 bp, meganucleases were engineered to target the mutated human XPC gene in *xeroderma pigmentosum* (Redondo et al., 2008), the human *RAG1* gene in severe combined immunodeficiency (SCID) (Grizot et al., 2009) or the HSV-1 virus (Grosse et al., 2011). The extraordinary effort required to retarget the recognition sequence of meganucleases is responsible for their drop in importance for genome editing. New approaches make use of the natural meganucleases in combination with auxiliary, programmable DNA binding domains, like TALE repeat arrays, ensuring utmost DNA cleavage specificity (Beurdeley et al., 2013; Boissel et al., 2014).

2.1.2. Zinc finger nucleases

Zinc finger nucleases were the first tools for genome editing exploiting a bipartite architecture consisting of a DNA-binding domain fused to a nuclease domain, usually *Fok*I (Kim et al., 1996; Bibikova et al., 2001; Chandrasegaran and Carroll, 2016).

The DNA-binding domain typically contains an array of three to six Cys_2-His_2 zinc fingers. Each finger is composed of about 30 amino acid residues in a conserved $\beta\beta\alpha$-fold and recognizes 3–4 base pairs of the DNA target by using four key amino acid residues. The combination of different zinc fingers allows to generate zinc-finger arrays capable of targeting nearly any desired sequence. The main drawback of the simple modular combination approach is that the specificities of individual zinc fingers are context dependent and usually overlap. To compensate the context-dependent interactions, alternative strategies like OPEN (Maeder et al., 2008) or CoDA (Sander et al., 2011) were developed to select for highly specific zinc finger arrays. Common zinc finger nucleases utilize the nonspecific catalytic domain of the type IIS restriction endonuclease *Fok*I. This domain does not contribute to binding specificity but has to dimerize for DNA cleavage (Bitinaite et al., 1998). To allow cleavage domain dimerization, two individual zinc finger domains must bind to opposite strands of the DNA in an inverted orientation usually separated by five to seven nucleotides (Fig. 3). To reduce off-target cleavage, it is essential to use engineered obligatory heterodimeric *Fok*I cleavage domains (Miller et al., 2007; Szczepek et al., 2007). A typical zinc finger nuclease, as depicted in Fig. 3, recognizing an 18 bp long sequence can confer specificity within 4^{18} base pairs, but targets are confined to sequences composed of triplets

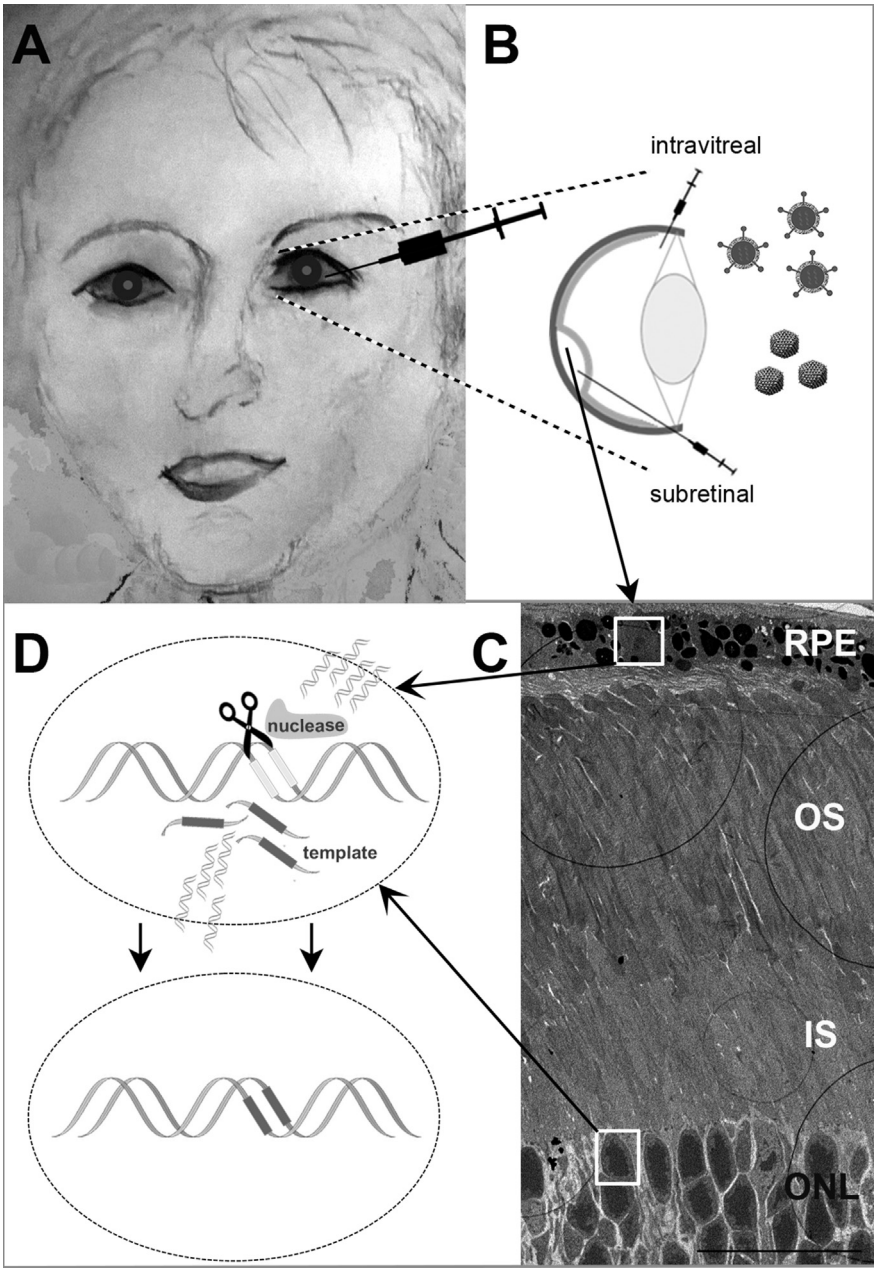

Fig. 2. Summary of key steps needed to be optimized to achieve successful genome editing in the retina. Initially, all necessary items for genome editing (i.e. endonuclease, template, modifying agents) need to be transferred to the target cells, which is usually realized by either subretinal or intravitreal injection of vector suspensions (**A, B**). Target cells in the retina are most often RPE and photoreceptor cells (**C**) (kindly provided by U. Wolfrum, Johannes Gutenberg-University, Mainz). Once, all items are expressed or present in the target cells, the DNA double strand break (DSB) is induced by the nuclease and repaired by use of the template (**D**). Nuclease and template will be eventually degraded, thus leaving nothing behind. Scale: 20 μm.

with corresponding zinc fingers resulting in a target probability approximately every 500 bp. However, zinc finger nucleases targeting a wide range of different genes have been constructed and successfully tested (Urnov et al., 2005; Carroll, 2008; Urnov et al., 2010; Rahman et al., 2011; Gaj et al., 2013).

Despite the modular structure of the zinc finger binding arrays, still great efforts are necessary to generate customized highly specific zinc finger nucleases with a reasonable off target activity (Schierling et al., 2012). Nevertheless, highly evolved zinc finger nucleases are the only genome editing tools that are currently tested as therapeutic agents in clinical trials for the treatment of Hemophilia B, Mucopolysaccharidosis I and HIV infections (Tebas et al., 2014) (www.clinicaltrials.gov).

2.1.3. TALEN (transcription activator-like effector nucleases)

TALEN closely resemble the zinc finger nucleases, but make use of an advantageous DNA binding module derived from transcription activator-like effector proteins of the bacterial plant pathogen *Xanthomonas*. These proteins are comprised of a central DNA binding region of tandem repeats. Each repeat usually is 34 amino acid residues long, with a nearly identical sequence, except amino acid residues at position 12 and 13, also referred to as repeat variable diresidues (RVD) (Scholze and Boch, 2011). Each repeat mediates the recognition of one base in a sequential manner with the specificity being determined by the RVD. In 2009, Boch et al. published this one repeat - one base relationship as “Breaking the Code of DNA Binding Specificity …” (Boch et al., 2009). The modular

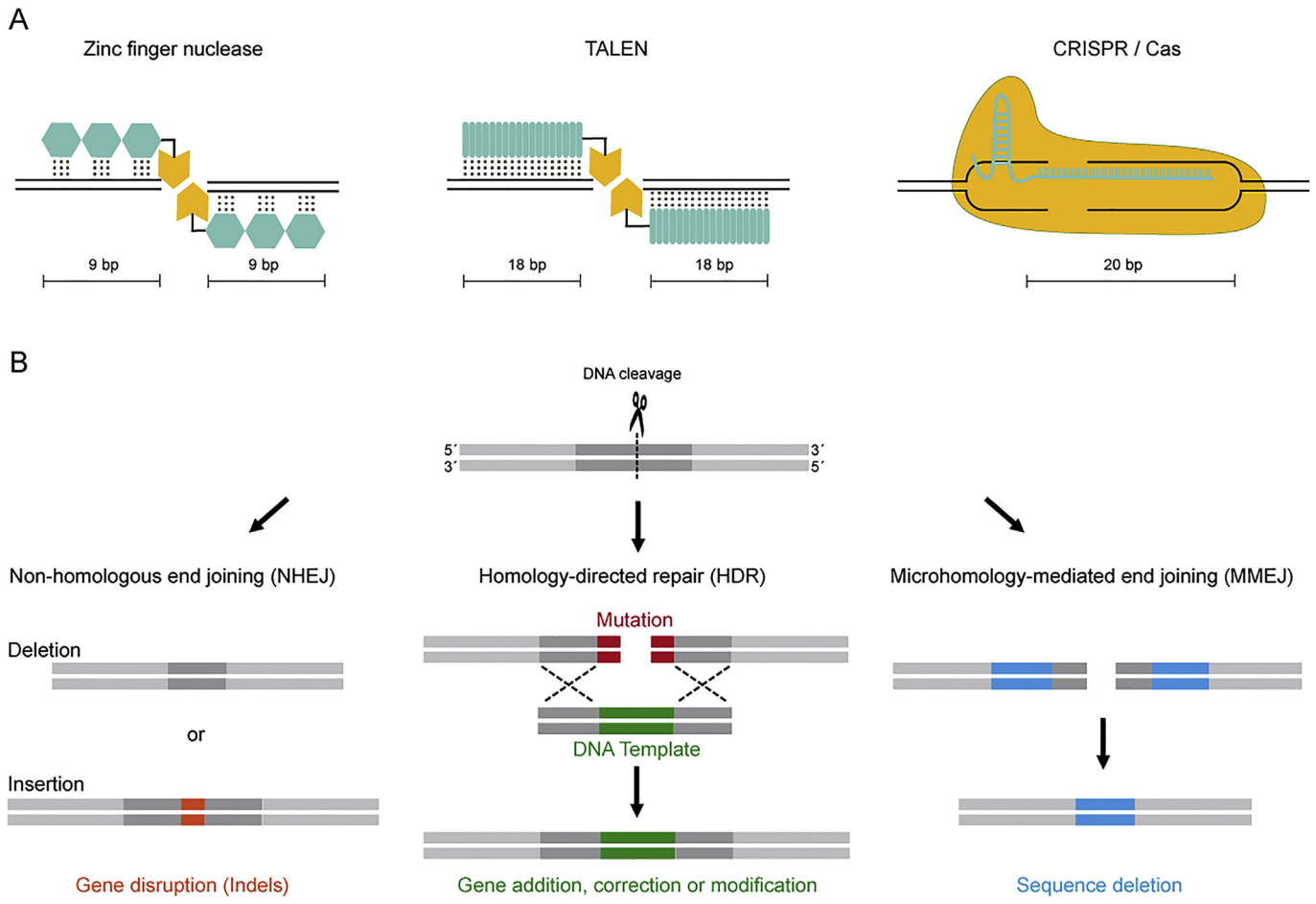

Fig. 3. Description of the currently most often used endonucleases and potential repair pathways. (**A**) Schematic drawing of the three currently used endonucleases. In turquoise, the DNA binding domains are represented, in yellow the DNA cutting proteins. The length of the DNA recognition sequence is depicted below each cartoon. (**B**) Three major different repair pathways exist to repair a DNA double strand break in eukaryotic cells. Each pathway is briefly described in the corresponding carton including the potential result of the DNA repair. Other mechanisms may also be involved.

architecture of the DNA binding domain, without considerable context-dependent interactions between the repeats, enabled the design of tailored DNA binding arrays from the scratch. For genome editing, the TALE array is combined with a nuclease domain, consequently replacing the zinc fingers (Fig. 3). Apart from the *Fok*I as a cleavage domain (Christian et al., 2010; Miller et al., 2011), other specific nucleases (Gabsalilow et al., 2012; Yanik et al., 2013; Boissel, Jarjour et al., 2014) or effector proteins (Maeder et al., 2013; Scott et al., 2014; Kungulovski and Jeltsch, 2015) can be fused to the TALE array, creating a universal platform for highly specific DNA targeting. Methods, like the "Golden Gate" cloning system (Cermak et al., 2011), solid-phase assembly methods (FLASH (Reyon et al., 2012),) or ligation-independent techniques (Schmid-Burgk et al., 2015) facilitated the efficient assembly of custom TALE arrays planed by web-based software (Doyle et al., 2012) and contributed to the increasing prevalence of TALE based genomic editing (Kühn et al., 2016). One disadvantage for the delivery of paired TALE nucleases is their size and their repetitive sequences, making it challenging to utilize viral systems (Holkers et al., 2013). Codon diversification between the repeats mitigated this issue and the delivery of TALENs with lentiviral and adenoviral vectors have been successfully demonstrated (Yang et al., 2013b; Holkers et al., 2014). For therapeutic approaches, one significant event in 2015 was the first-in-man application of TALEN engineered universal CAR19 T-cells in case of a pediatric acute B lymphoblastic leukemia (Qasim et al., 2015).

2.1.4. *CRISPR/Cas*

In the last century, restriction endonucleases, which represent a defense mechanism of bacteria against invading viruses, led to the development of recombinant DNA technology (Roberts, 2005). Today, another prokaryotic adaptive immune system (Gasiunas et al., 2014), in particular the Cas9 nucleases belonging to the CRISPR system type II, revolutionizes genome editing (Doudna and Charpentier, 2014). Distinct from the other genomic editing tools, the Cas9 endonuclease is guided via an RNA molecule to its target site (Fig. 3). Consequently, genome editing can be easily directed to virtually any genomic site by delivering the complementary RNA along with the Cas9 endonuclease.

In bacteria, the CRISPR system cuts out small fragments (~20 bp) from invading virus or plasmid DNA and integrates them as protospacer between short palindromic repeats in the CRISPR array. Subsequently, the repeat CRISPR array is transcribed and the RNA fragments (crRNA) corresponding to the individual integrated foreign DNA fragments will be excised and integrated in the Cas9 endonuclease with the help of a bacterial trans-activating crRNA (tracrRNA). This activated Cas9 endonuclease can specifically cleave DNA sequences complimentary to the crRNA only if an additional 3–5 bp long sequence, the PAM (protospacer adjacent motif), is present at the 3'-end of the target. The PAM sequence is recognized only by the Cas9 protein and is not present in the repeats of the CRISPR array, preventing the cleavage of the bacterial DNA (Horvath and Barrangou, 2010).

In 2012 the labs of Doudna and Charpentier demonstrated that crRNA and tracrRNA can be combined to one guide RNA (gRNA) and that engineered gRNA can stimulate targeted DNA cleavage in vitro (Jinek et al., 2012). The first genome editing of mammalian cells using the CRISPR/Cas system was described shortly after (Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013b). Since then, an enormous number of publications demonstrate that CRISPR/Cas technology was successfully applied for genome editing in an increasing number of organisms including crops, fruit fly, zebrafish, frog, monkey, mouse, rat, pig and human.

The CRISPR/Cas technique is extensively used *ex vivo* to target genes for gene disruption, addition or correction in all kinds of cells comprising embryonic stem cells or zygotes to generate animal models (Mei et al., 2016). One particular feature of the CRISPR/Cas system is to target simultaneously several sites in the genome by using multiple gRNAs (Cong et al., 2013). This potential was exploited e.g. by the Church lab for a genome-wide inactivation of 62 porcine endogenous retroviruses to reduce transplant immunogenicity for xenotransplantations (Yang et al., 2015). Reports of somatic gene editing of adult animals by CRISPR/Cas, using viral vectors or hydrodynamic injection for delivery, are still rare (Platt et al., 2014; Xue et al., 2014; Swiech et al., 2015; Wang et al., 2015; Weber et al., 2015; Nelson et al., 2016; Tabebordbar et al., 2016).

2.1.5. Off-target analysis

Since the first genome editing studies were published, the concern of off target toxicity was eminent. If the endonuclease is not sufficiently specific for the desired target site, DSBs can be induced at other locations within the genome, thus leading to toxic effects and death of the cells. Initially, this toxicity was studied by cell viability assays such as the propidium iodide cytotoxicity assay, in which a higher number of stained (i.e. death) cells indicate increased toxicity of the tested endonuclease (Greenwald et al., 2010). Also, viability was studied in cells expressing GFP and transfected with the nuclease of interest. After 3–5 days, the number of living cells was counted by FACS and compared to non-transfected cells (Yanik et al., 2013). If the number of GFP positive cells was significantly lower compared to controls, the nuclease caused a toxic effect to the cells. More recently however, the potential of next generation sequencing was discovered to examine off-target toxicity, such as by the use of the guide seq method to study off-target site for the CRISPR/Cas9 system (Tsai et al., 2015).

One constrain for the CRISPR/Cas cleavage is the presence of the correct PAM in the target DNA. Most studies take advantage of the first described Cas9 nucleases from Streptococcus pyogenes, recognizing the PAM sequence NGG. Nowadays, Cas9 nucleases from other origins (Ran et al., 2015; Haeussler and Concordet, 2016) or with newly engineered PAMs (Kleinstiver et al., 2015) are available, extending the repertoire of potential target sequences. Selecting the right target sequence and the corresponding gRNA is crucial for efficient and specific cleavage. Web-based resources predict gRNA efficiency and also potential off-targets (Doench et al., 2016; Haeussler and Concordet, 2016). Moreover, truncated gRNA with only 17 or 18 nucleotides of complementarity cleave the intended target site with higher efficiency and reduce off-target effects (Fu et al., 2014). Also other strategies to reduce genome-wide off-target cleavage of the Cas9 nuclease have been described: using pairs of Cas9 nickases with only one active center (Mali et al., 2013a; Ran et al., 2013), pairs of catalytically inactive Cas9 nucleases fused to the non-specific *Fok*I nuclease (Guilinger et al., 2014; Tsai et al., 2014) or rationally engineered high precision Cas9 nucleases (Kleinstiver et al., 2016; Slaymaker et al., 2016). It seems that the Cas9 protein alone is not harmful for cells, as shown by the Zhang lab, producing a CRISPR-Cas9 knockin mouse (Platt et al., 2014), with no obvious abnormal health effects after 20 generations.

Despite recent advances to avoid off-target cleavage, which can cause unwanted gene mutations or chromosome rearrangements, an unbiased sensitive whole-genome off-target analysis (Yee, 2016) in the targeted cell type is indispensable to evaluate candidate nucleases for clinical applications (Corrigan-Curay et al., 2015).

With the improved CRISPR/Cas systems and TALENs we now have powerful tools for genome editing, shifting the bottleneck on the way to the clinical applications to safe and effective delivery vectors and the promotion of the intended repair pathway.

2.2. DNA repair mechanisms

There are two main pathways in DNA double strand repair break: HDR and NHEJ. Furthermore, NHEJ is divided into classical NHEJ (C-NHEJ) and alternative NHEJ (A-NHEJ), also called microhomology-mediated end joining (MMEJ). NHEJ takes place during the whole cell cycle, whereas HDR is only present in S- and G2-phase, when the sister chromatid can act as a template (Lieber, 2008). Therefore, the cell cycle phase and CDKs (cyclin-dependent kinases) play crucial roles in the pathway choice decision (Ceccaldi et al., 2015). Frequently, a double strand break occurs following ionizing radiation or indirectly through chemical modifications causing alterations of the replication fork (Pfeiffer, 2000). By employing endonucleases, targeted DSB can be introduced in a therapeutic setting (Jasin and Haber, 2016). A short description of DSB recognition and the different repair pathways is given in the following section (Fig. 4).

Initially, the DSB is recognized and bound by ATM (Ataxia telangiectasia mutated) and ATR (Ataxia telangiectasia and Rad3-related protein). These serine/threonine-kinases lead to a fast phosphorylation of H2AX, then called γH2AX. H2A is one of the five major histone families organizing the eukaryotic DNA into chromatin. γH2AX can be used as a DSB marker. It plays a crucial role in recruiting checkpoint factors and others to the DSB, like MDC1 (Mediator of DNA damage checkpoint protein 1) (Yuan et al., 2010). Through binding of the mediator protein MDC1, the E3 ubiquitin protein ligase RNF8 (ring finger protein 8) is activated. This leads to the ubiquitinylation of H2AX. The reaction is also enhanced by RNF168 (ring finger protein 168) (Brandsma and Gent, 2012). It results in recruitment of 53BP1 (p53 binding protein 1) and RAP80 (receptor-associated protein 80) (Stewart et al., 2009).

RAP80 binds BRCA1 (breast cancer 1), which leads either to MMEJ or HDR. In contrast, 53BP1 results in binding factors, which are part of the NHEJ pathway (Ward et al., 2003; Ginjala et al., 2011). These two molecules, BRCA1 and 53BP1, are known to form foci at DSB sites (Manis et al., 2004). The current understanding is that proteins from both repair pathway machineries compete for the free DNA ends. How the decision is made by the cellular repair machinery for a given pathway is currently largely unknown. Important factors in the decision making process represent the cell cycle stage and the presence of a template DNA.

2.2.1. Non-homologous end joining

NHEJ is responsible for fast DSB repair. The ends of a DSB are directly ligated, which can lead to small insertions, deletions or substitutions. Hence, NHEJ is generally deemed to be an error-prone DSB repair pathway. It starts by binding of the Ku70/Ku80 heterodimer at sites which are marked by DNA damage sensing proteins (Fig. 4). Ku is a prominent protein in the human proteasome (about 400,000 molecules per cell) and has a strong affinity to DNA ends, which are consequently stabilized. The heterodimer forms an asymmetric ring, interacts with the DNA backbone and therefore encloses the double stranded DNA independently of the

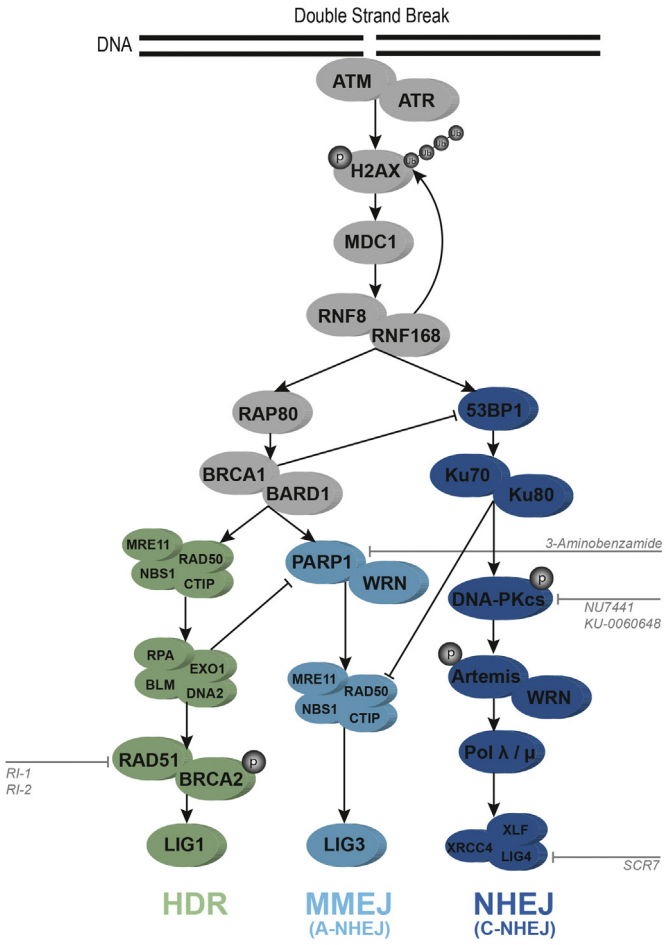

Fig. 4. Description of the proteins involved in the different repair pathways and their sequential appearance at the target site. Upon a DNA double strand break, DNA damage sensing proteins (enveloped in grey) are attracted to the site and process the DNA ends. During this stage, the decision of a given repair pathway very likely takes place. Eventually, either homology directed repair (HDR, proteins in green), microhomology mediated endjoining (MMEJ, proteins in turquoise, also called alternative (a) NHEJ), or non-homologous endjoining (NHEJ, proteins in blue, also called classic (c) NHEJ) will be lounged to repair the DSB. Inhibitors of certain proteins are highlighted in grey with a grey line pointing to the target molecule.

DNA sequence (Blier et al., 1993; Falzon et al., 1993). Ku70/Ku80 then recruits DNA-PKcs (DNA-dependent protein kinase catalytic subunit). Together they form a huge complex, named DNA-PK. The DNA end processing is regulated through the autophosphorylation of DNA-PKcs (Chan et al., 2002). Subsequently, the protein is inactivated and dissociates from the DNA. Furthermore, WRN (Werner syndrome ATP-dependent helicase) and the nuclease Artemis are phosphorylated and activated (Karmakar et al., 2002). They are responsible for the DNA end processing. After completing this process, the polymerases λ and μ add missing nucleotides (Fan and Wu, 2004; Schipler and Iliakis, 2013). The final step is the ligation of the DNA DSB ends via the ATP-dependent LIG4 (DNA Ligase IV), XRCC4 (X-ray repair complementing defective repair in Chinese hamster cells 4) and XLF (Nhej1, nonhomologous end-joining factor 1) which form a complex (Povirk, 2012).

2.2.2. Microhomology-mediated end joining

The alternative pathway to the classical NHEJ is MMEJ, for which still some mechanistic details remain unclear. In contrast to NHEJ, the ends of the DSB are linked via microhomologous domains on both sites (5–25 nucleotides) of the DSB, which can lead to a deletion at this position (Deng et al., 2014). So far, not much is known about specific features these sequences have to contain, but it is considered that homology needs to be 100%. Interestingly, if a template DNA is present containing the microhomologous regions, precise genome editing is possible. MMEJ is Ku- and Ligase 4-independent, occurs mainly during the S-phase of the cell cycle and competes with HDR. The MRN-complex, including Mre11 (MRE11A homolog A), Rad50 and Nbs1 (Nibrin), together with CtIP (C-terminal binding protein (CtBP)-interacting protein) are responsible for the DNA end processing (Symington and Gautier, 2011). Therefore, these proteins are indispensable for MMEJ just as for HDR. The proteins process a small number of base pairs and make the DNA ends available for MMEJ (Truong et al., 2013). The ends are ligated via DNA ligase 3. The parameters leading to MMEJ instead of HDR and NHEJ are still unclear (Sinha et al., 2016).

2.2.3. Homology directed repair

In contrast to NHEJ, HDR is a nearly error-free process, because of the specific template used for an accurate repair (Heyer et al., 2010). After recognition of the DSB through DNA damage sensing proteins (Fig. 4), the MRN complex is recruited. Single stranded DNA 3'-overhangs are generated through this complex collaborating with EXO1 (exonuclease 1), DNA2 (DNA replication helicase 2 homolog) and CTIP, which was found in budding yeast (Symington and Gautier, 2011). This process is also called DNA end resection. The generated overhangs are crucial for the efficient repair. A region, which is homologous to the overhang, is used as a template for the repair. The processing of the DNA at the DSB is in addition controlled by BRCA1, a well-known tumor suppressor protein (Daley and Sung, 2014). Subsequently, BLM (Bloom syndrome, RecQ helicase-like) and EXO1 recruit RPA1 (replication protein A1). RPA1 rapidly binds single stranded DNA, stabilizes the ends and inhibits the formation of secondary structures (Mimitou and Symington, 2009). After that, RPA1 gets replaced by RAD51. This protein searches for homologous regions and promotes together with BRCA2 (breast cancer 2) the accumulation of DNA. Finally, the ligation of the DNA ends is made by LIG1 (DNA ligase I) (Holthausen et al., 2010; Yan et al., 2011).

2.2.4. Alternative strategies to edit the genome

While NHEJ is often favored over HDR, especially in human cells, and because NHEJ is error prone and can lead to mutations or chromosomal rearrangements, it is important to be able to control the DNA repair mechanisms or, in the case that HDR of MMEJ is wanted as editing approach, find strategies that most likely do not employ NHEJ as repair mechanism (Karran, 2000). NHEJ could, in turn, be employed to knock-out toxic gain-of function alleles in autosomal dominant diseases.

One way would be to inhibit NHEJ factors by siRNA technology or specific blockers (see examples in Fig. 4), which was shown to result in increased activities of HDR proteins to repair the DSB, a mechanism further demonstrating the competitive interaction of the different proteins at the DSB site (Budke et al., 2012; Munck et al., 2012; Chu et al., 2015; Maruyama et al., 2015; Tadi et al., 2016). It remains to be seen how useful this approach may be in *in vivo* applications, where certain drugs may be toxic and therefore cannot be employed or where siRNA technology may not be amenable either.

Another possibility is a site-directed single strand break (SSB), also called nick. Recent work has shown that the cleavage of only one DNA strand leads to less off-site targeting compared to a DSB (Metzger et al., 2011). In most cases, the SSB gets repaired by a very fast global repair process. This process is divided into four steps, similar to the DSB repair: SSB detection, DNA end processing, DNA gap filling and DNA ligation. PARP1 (poly(ADP-ribose) polymerase

1) plays an important role in detecting the SSB (Caldecott, 2014). Nicking Cas9 variants with less toxicity were already mentioned in chapter 2.1.4. Nicking homing endonucleases are a good alternative due to their small size compared to TALEN or CRISPR/Cas. A comparison between the wt homingendonuclease I-*Ani*I (inducing a DSB) and a nicking variant of I-AniI showed lower HR ratios for the nicking nuclease by a factor 4 to 6 and also lower mutation rate by a factor of 150. No severe toxicity was observed (Davis and Maizels, 2011; Metzger et al., 2011).

More recently, a different way of changing a given DNA sequence has been introduced, the targeted base editing (Komor et al., 2016). This technology, a fusion between an inactivated Cas9 protein and a cytidine deaminase allows to change one base into another (here: C to T) without previous introduction of a DSB or the use of a template, thus potentially avoiding the complications associated with DSB repair, in particular the generation of indels. However, if the treatment goal is to insert or remove bases from the sequence, this technology cannot be employed.

2.3. Template

The efficiency of HDR is determined by many parameters, such as the cell cycle, the cell type, chromosomal region, the activity of endogenous repair system, and the DNA donor template. During the S and G2 phases of the mitotic cell cycle, the homologous sequence is naturally present as sister chromatid (Heyer et al., 2010). However, template DNA can also be engineered artificially and provided exogenously for repair. For accurate DNA repair by HDR, an undamaged donor template with sufficiently long homology regions flanking the target site is required. Although chemical or genetic regulation of repair pathway components and manipulation of cell cycle can bias the repair outcomes toward HDR (Srivastava et al., 2012; Chu et al., 2015; Maruyama et al., 2015), it may be undesirable for therapeutic applications because they can disturb the function of cellular genes and lead to tumor formation. Recently, up to 60% HDR rate in HEK cells has been reported by using Cas9 or nickase variants and single-stranded DNA (ssDNA) donors in the absence of chemical and genetic intervention (Richardson et al., 2016). It demonstrates that designing DNA donor in an appropriate manner plays a central role in increasing HDR frequencies.

Linearized or double-stranded DNA plasmid sequences, as well as ssDNA oligonucleotides, are used as template for homologous recombination at the target site (Fig. 5). The size of the intended sequence changes, the length of the homology arms, and the insertion site of the mutation are important factors to be considered (Carroll and Beumer, 2014). Although the exact mechanism by which donor design increases HDR frequencies is still under investigation, several evidences have shown its influence on gene targeting outcomes.

In mammalian cells, total homology up to 10 kb increases the HDR frequencies without even a target cleavage, an observation that has been widely used for creating gene-targeting vectors for the generation of animal models (Deng and Capecchi, 1992). In the presence of an induced DSB at the target site, a plasmid donor with at least 1–2 kb of total homology is recommended for large sequence changes more than 100 bp (Dickinson et al., 2013; Yang et al., 2013a). The cleavage site should be as close to the insertion site of the mutation as possible, ideally less as 10 bp away. The efficiency of recombination increases as the length of homology arms increases, while the efficiency decreases as the size of the DNA insert increases (Li et al., 2014). Although linear donor template has

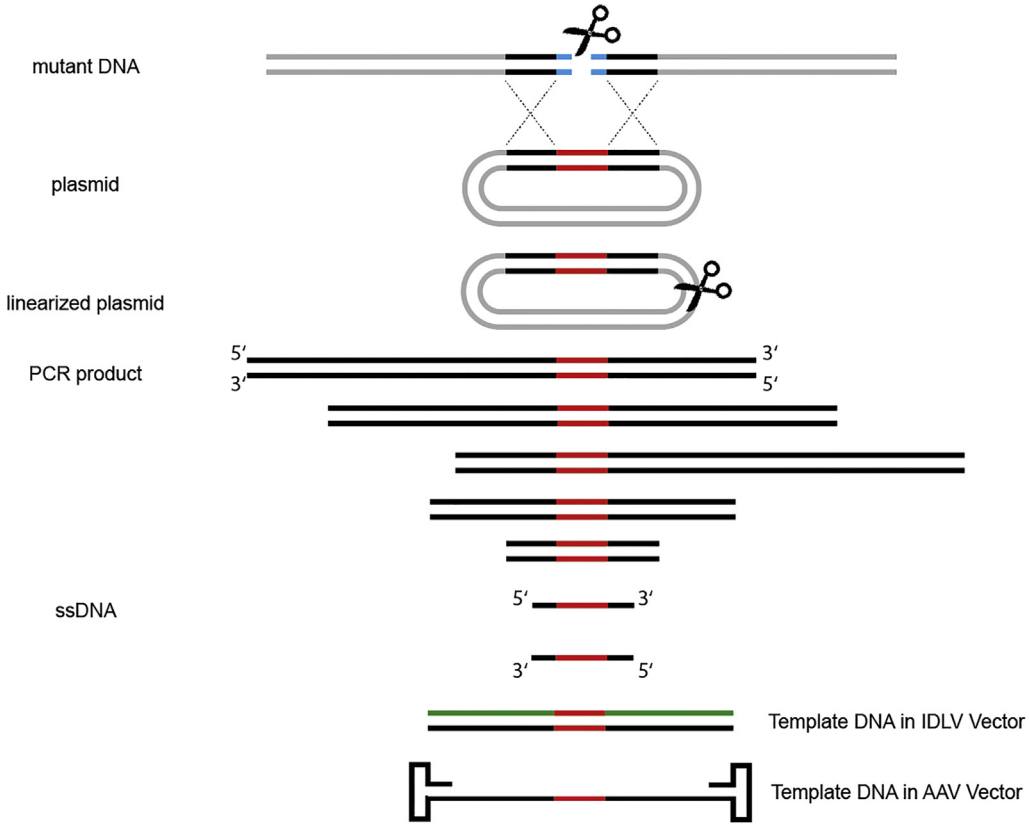

Fig. 5. Design of currently available templates. Templates can come as double stranded plasmid, linearized plasmid, PCR product, single stranded (ss)DNA or viral vector DNA (AAV or IDLV).

been shown to yield higher HDR frequencies compared to the circular donor (Carroll, 2011), the higher degradation rate and undesirable spontaneous integration could be critical for genome stability. SsDNA sequences are usually more efficient than plasmid donors for small sequence changes up to 50 bp; 30–80 bp of homology arms on each side flanking the target is preferred (Sakuma et al., 2016).

One important point to note is that Cas9 behaves differently from ZNFs and TALENs. SsDNA donors complementary to the non-target strand showed higher HDR frequencies due to the asymmetric release of Cas9 from the cleaved DNA strand (Richardson et al., 2016).

2.4. Vectors for gene transfer

Different delivery systems are available to transfer genes into the retina. Differences are present with regard to longevity of transgene expression, immune response by the host organism and size of the transferred genes, among others (summarized in Table 1).

In order to perform *in vivo* genome editing, an expression cassette containing the genetic information encoding the nuclease together with a promoter sequence and a polyadenylation signal needs to be transferred into the target cell. In case the CRISPR/Cas system is used, an additional expression cassette containing the guide RNA information with a promoter sequence (usually about 400bp in size) is also needed. If HDR or MMEJ in a therapeutic setting is envisaged, a template DNA (ranging from 100 to more than 1000 bp in size, depending on the target sequence to be repaired) is crucial and needs to be transferred as well. The efficient transfer of these three items represents one of the major hurdles to overcome in *in vivo* retinal genome editing, and the choice of an optimal vector system is crucial.

Many reviews exist that highlight the different characteristics in general (Gaj et al., 2015; Nelson and Gersbach, 2016). Here, we describe the characteristics of the most promising vectors for genome editing purposes in the retina *in vivo*.

2.4.1. AAVs

Adeno-associated virus (AAV) is a mammalian single stranded DNA parvovirus. Its genome consists of about 4.7 kB containing two inverted terminal repeats (ITRs) flanking genes for replication (rep) and building the virus capsid (cap). AAVs are known to infect non-proliferating and proliferating cells (Baba et al., 2012). After infection of the host cell by receptor mediated endocytosis, the virus genome persists as episome in the cell. In order to generate a recombinant vector, the entire virus sequence except the flanking inverted terminal repeats are replaced by the expression cassette of choice. Several recombinant AAV (rAAV) serotypes which are able to target different cell types have been extensively characterized. While the AAV serotype 2 was employed as photoreceptor (PR) and retinal pigment epithelium (RPE) transducing vector for many years, the serotypes 5, 8 and 9 have more recently gained higher importance as transfer vectors to the retina.

Unlike any of the other vehicles which can be used for genome editing, AAVs were shown to stimulate homologous recombination in mammalian cells (AAV-mediated gene targeting) by its own presence in a very efficient way (Gaj et al., 2015). Up to 1% HDR could be shown in human fibroblasts using AAVs without introduction of a DSB (Russell and Hirata, 1998). The presence of a DSB increases the efficiency by up to 1000 fold (Russell and Hirata, 1998; Gaj et al., 2015). One of the possible reasons for the high HDR efficiency of AAVs is the single stranded nature of the AAV template. Other potential factors are the nuclear localization of a high number of genomes and the recombinogenic ITRs (Hirata and Russell, 2000). It is possible to improve the efficiency rate of AAV-mediated gene targeting by increasing the homology arm length, finding the best length of homology and locating the target sites within the transcriptional units (Gaj et al., 2015). Other important aspects as type of mutation and chromosomal context are more difficult to influence (Hirata and Russell, 2000; Hirsch et al., 2010). When used together with ZFN, rAAV2 based HDR donor templates resulted in a frequency of 6% HDR in human cells (Händel et al., 2012).

Though AAVs are promising vehicles for gene transfer, one main disadvantage is their maximum cargo capacity of 4.7 kB, which is not sufficient for some of the nucleases. For example, the currently used Streptococcus pyogenes (Sp) Cas9 is about 4.2 kb in size and together with the promoter sequence and its gRNA, it is too large to fit into a single AAV vector. Therefore, one would need two AAV vectors (one for the Cas9 gene, and one for the guide RNA sequence), or one of the other transfer vectors in order to transfer the genetic information into the target cells. In contrast, the smaller *Staphylococcus aureus* (Sa) Cas9 is about 3.2 kb in size and thus, can be transported together with the guide RNA by one AAV vector alone (Gaj et al., 2013; Friedland et al., 2015; Ran et al., 2015).

To transfer larger expression cassettes, dual vectors have been developed, which are based on the ability that AAVs can concatamerize by splicing, HDR, or a combination of both (Trapani et al., 2014a). These trans-splicing vectors have a lower expression rate than single AAVs (Maeder and Gersbach, 2016), showing only around 16% protein expression in HEK293 of that transduced with a single AAV (Xiao et al., 2000). However, mouse models of Stargardt macular dystrophy and Usher syndrome 1B treated with these vectors showed a milder phenotype (Trapani et al., 2014a). The dual vectors were also tested in large cone-enriched pig retinae, where the transfer-efficiency was significantly higher than in mice, but still two-to threefold lower than the transduction rate achieved by a single AAV vector (Colella et al., 2014). To further increase the transduction efficiency, ITRs with homology to F1 phage seems to be promising (Trapani et al., 2015). Beside mouse and pig model systems, rAAVs were used to transduce ON bipolar

Table 1
Vectors used for retinal gene transfer.

	AAV	LV	Supercharged proteins	Nanoparticles
Capacity (kB)	4,8	8	?	14(eye)-20(liver)
Size	20 nm	120 nm	15-30 kDa	8-25 nm
Genome	ssDNA	ssRNA	–	–
Integration	No	Yes	No	No
Clinical trails	12	4	–	–
Target cell (Cell specificity)	Dividing and non dividing	Dividing and non dividing	Dividing and non dividing	Dividing and non dividing
Cell entry	endocytosis	endocytosis	endocytosis	endocytosis
Nuclear entry	yes	yes	yes	yes
Immunogenicity	low	low	?	low

cells in retinal explant cultures of post mortem human eyes, which was not of great success (Fradot et al., 2011).

Altogether, AAVs are promising vehicles for genome editing despite their low cargo capacity. They show no immune response when subretinally injected and are able to express genes in non-dividing cells like photoreceptors and other neurons.

2.4.2. Lentivirus-based vectors

Lentiviruses are retroviruses with a ssRNA genome that is integrated into chromosomes of target cells. Because of this random integration, LVs are not as safe as non-integrating vehicles, but able to express transgenes for a very long time, especially in constantly dividing cell populations. To overcome the random integration issue, an integrase deficient lentiviral vector (IDLV) was developed (Philpott and Thrasher, 2007). IDLVs have been used in studies with different human target cells in the context of genome editing. They seem to be promising vehicles for donor templates in dividing cells, generating HDR in up to 10% of a given cell population (Chen and Gonçalves, 2015). Another possibility to ensure that there is no long term expression of the genome editing tools, which may lead to off-target activity, is to integrate cleavage sites for the used cutting enzyme in the vector genome.

One big advantage in contrast to AAVs is that LVs can carry large transgenes up to 8 kb (Trapani et al., 2014b), although larger transgenes may yield lower functional particle titers (Kumar et al., 2001). This characteristic makes LVs suitable to be used to transfer all kinds of nucleases, including SpCas9 or TALEN fused to restriction enzymes (Fok-I). However, if a large template is needed for HDR, a second LV may still be needed. Subretinal injection of lentiviral vectors pseudotyped with the VSV-G (vesicular stomatitis virus glycoprotein) results in a strong transgene expression in RPE cells and to a lower extend in PRs. Differences regarding the transduction efficiency in retinal explant cultures from mice and human were observed, potentially indicating that transduction rates may be species specific (Lipinski et al., 2014).

Since most of the LV pseudotypes are known to transduce mainly RPE cells in adult retina, Puppo et al. tested 7 LV pseudotypes for PR transduction. They found LV-GP64 to transduce PR, but still AAV2/8 achieved a higher efficiency (Puppo et al., 2015). Furthermore, LVs are promising vehicles to transfer genes into iPSCs because of their stable integrating capability. This was already shown in a study, in which an expression cassette containing the correct CEP290 cDNA was stably introduced into iPSC-derived, photoreceptor precursor cells from Cep290-associated LCA patients, leading to a stable expression of wt CEP290 protein (Burnight et al., 2014).

Altogether, LVs have the potential to be used as transfer vectors for endonucleases and template in treatment approaches for retinal diseases, even though transduction efficiency in PR is limited.

2.4.3. Supercharged proteins

For genome editing purposes, especially using CRISPR/Cas9, direct transfer of the Cas9 proteins could be of interest, because there is no ongoing activity of the cutting enzyme needed, or even undesired due to their potential off target toxicity. However, simple protein transfer into cells *in vivo* is very inefficient. One way to increase transfer efficiency is to change the net charge of proteins making them capable of traversing lipid membranes.

Proteins with very high theoretical positive or negative net charge are called supercharged proteins. Such proteins are known to be able to penetrate mammalian cells in vitro and *in vivo*. Reporter proteins like GFP, streptavidin and glutathione S-transferase have been modified to act as supercharged proteins. These proteins can bind to small molecules like DNA or to other proteins, which results in an electrostatic complex. Like AAVs and LVs, supercharged proteins penetrate cells by endocytosis. Thompson et al. described that superpositively charged proteins that have a greater property to transduce mammalian cells than cationic proteins (Thompson et al 2008). Cronican et al. tested supercharged GFP molecules (+36 net charge) in adult mouse retina by subretinal injection and observed a GFP signal in the entire retina. They also analyzed if supercharged GFP could transport active Cre recombinase in mouse p0 retina and showed a small amount of functional enzyme to be delivered into the retinal cells (Cronican et al., 2010).

More recently, another group reported that negatively charged proteins, containing anionic domains can also be used as vehicles for protein transfer *in vivo*. They showed 20% genome modification after transduction of Cas9:sgRNA complexes into mouse inner ear hair cells (Zuris et al., 2015). Nonetheless, nuclear localization and target cell specificity need to be further improved regarding supercharged proteins, before such an approach may reach clinical stage.

2.4.4. Nanoparticles

Nanoparticles (NPs) act like DNA-histone complexes, containing positively charged peptides, bound to a single DNA molecule. They enter the target cell by binding to the nucleolin receptor and are transported directly into the nucleus (Chen et al., 2008). A big advantage of nanoparticles is their capacity to carry up to 14 kB when used as vehicle in the eye (Han et al., 2012a, b), which would make them suitable for the transfer of nucleases, guide RNAs and template all in one vector. Peptides for ocular delivery (POD) can enter RPE and PR cells *in vivo* after subretinal injection. Johnson et al. created a POD-GFP fusion protein and localized it in the nucleus, therefore POD seems to act as an nuclear localization signal (Johnson et al., 2010). The same group showed a transduction efficiency of 40% of the retinal surface in the ONL.

In direct comparison to AAVs, NPs, which are composed of polyethylene glycol-substitutes polylysine (CK30PEG) showed matchable scale and longevity *in vivo* in mouse retina. Although NPs are less efficient per vector genome, the gene expression level is similar to AAVs (Han et al., 2012a, b). Nanoparticles are able to transduce dividing and non-dividing cells due to their transport mechanism (Koirala et al., 2013a), the transferred genes remain episomal and show no toxicity in PR and RPE cells (Han et al., 2012a, b). In 2012, Han et al. were able to deliver the ABCA4 cDNA with a size of 6.8 kb packed in NPs (CK30PEG) into Abca4-deficient mouse PRs. The group showed an expression of the ABCA4 protein for up to 8 month post injection (Han et al., 2012a, b). Another study showed the possibility to treat RPE cells with a gene of interest which was too large for AAVs (Koirala et al., 2013b). These studies demonstrate the big advantage of NPs in comparison to AAVs, regarding the size of the gene transferred.

Another positive characteristic is that compared to AAVs, NPs express packed genes faster. To specify the cells which express the gene transported by NPs, a specific promotor can be used, for example rod and cone mouse opsin promoter (MOP) (Han et al., 2012a, b). The therapeutic effect of the wt peripherin2 gene packed in NPs were tested in a retinitis pigmentosa mouse model (rds+/-) at P5 and P22. The rod function was improved after treatment and cone function showed even wt level. The authors concluded that NPs are able to transduce mitotic and postmitotic PRs (Cai et al., 2010). Differences could be detected regarding the types of injection. If ultrapure oligochitosans carrying pCMS-EGFP were subretinally administrated, RPE and PRs were transduced, while intravitreal injection caused mainly ganglion cells and some PR transduction in rat retina (Puras et al., 2013a, b).

Taken together, nanoparticles represent a promising technology to transfer genes and template to the retina, especially because of the unlimited size of DNA particles to be transferred and the

temporarily limited nature of the transgene expression.

In conclusion, several gene transfer systems are present to deliver the genetic information that encodes for the endonuclease together with the template DNA. While AAV vectors, currently considered the gold standard for retinal gene transfer, may have significant limitations to transport both the genetic information of the endonuclease and any potential template, theses hurdles can potentially be overcome by use of other viral vectors or nanoparticles, each having its own limitations. A combined delivery of the endonuclease as protein and the template within an AAV vector may be the optimal choice, since protein delivery ensures a temporal burst of endonuclease activity while AAV delivery increases DNA repair machinery activity by itself.

3. The retina

3.1. Cell culture vs tissue – intermediate model?

Cell cultures are an ideal model to study cellular pathways and alterations of the cellular machinery since these cells continue to divide for a long time and are easily modifiable in terms of transferring genetic material and observing metabolic changes. Therefore, cell culture technologies have been applied to study DNA repair mechanisms since early on. In fact, most of the current knowledge about these mechanisms is based on data obtained from cell cultures. Not surprisingly, knowledge about the cell cycle dependent activities of the different repair mechanisms relies on the capacity of scientists to analyze cells from cell culture systems at defined stages of mitosis. Not to mention that literally all cells in a dish are exactly similar, as they represent the result of clonal amplification of a single cell.

When it comes to post-mitotic neurons in general, almost nothing is known concerning the activity of DNA repair machineries, expression and localization of proteins involved in chromatin structure, DNA damage sensing and cell cycle reactivation. This gap in the currently available knowledge represents a significant hurdle in the further development of genome editing strategies for post-mitotic tissues in general and certainly for approaches regarding the retina.

Since the retina is a highly structured organ with at least 5 different types of neurons (photoreceptors, bipolar cells, horizontal cells, amarcrine cells, ganglion cells) and macro- and microglia cells, it is difficult to generate valid information about the DNA repair status in each cell type separately. With over 80%, photoreceptors represent the vast majority of retinal cells, thus making the analysis of whole retina lysates meaningful if one considers potential gaps from the remaining cell populations.

Not only the DNA repair pathway situation (reviewed in the next section) is difficult to assess in retinal tissue, the issue of gene or protein transfer to the target cells within the retina remains crucial in the further development of such a treatment approach. In order to study the impact of genome editing approaches on the different retinal cells, a good model is needed. That is, apart from *in vivo* models, to have a system that allows to easily administer different molecules to increase or block certain repair pathways, to manipulate the macroenvironment within the tissue (e.g. inducing hypoxia) and observing alteration to the retinal morphology over time.

In our view, the organotypic retina culture system represents an ideal intermediate system between the cell culture and the complex *in vivo* situation. Such a system was first described more than a decade ago (Caffe et al., 2001; Hatakeyama and Kageyama, 2002; Pang et al., 2004) and usually keeps the retina in a viable state for at least 10–14 days (Fig. 6). Organotypic retina cultures display much of their *in vivo* phenotype in terms of cytoarchitecture and stratification assuming that intercellular connections are preserved as well. The post mitotic character of all different neuronal cell types is preserved and thus, can be studied in detail. Cell type specific targeting can be investigated by application of vectors onto the photoreceptor side or the ganglion cell side mimicking subretinal or intravitreal injections. In normal neonatal mouse retinae, the cell transduction properties of AAV1, AAV2 and AAV5 vectors were compared side-by-side both *in vivo* and in vitro (Pang et al., 2008). It was shown at least in the neonatal retina culture that AAV vectors behave analogously in organ culture and *in vivo.* In mature retina culture systems, the transduction efficiency is comparably low, but research is currently undertaken in order to increase gene transfer efficiency (Fig. 7). Therefore, the organotypic retina culture represents a viable alternative to *in vivo* assays.

An alternative model system represents the 3D retina culture (also called organoids) started from iPSCs that eventually form retinal organoids and reach a layer architecture somewhat similar to that found in adult retina (Clevers, 2016; Kaewkhaw et al., 2016). However, in depth analysis of the mitotic state of the cells within such a culture system needs to be done before it may be used to model *in vivo* genome editing approaches.

3.2. DNA repair activity in post-mitotic photoreceptors

With the murine retina being a tissue directly exposed to UV light (mice possess UV sensitive cones), many people would argue that the DNA repair machinery is highly active in these cells, given the fact that the repair of DSBs induced by radiation is crucial for the maintenance of genomic stability. In contrast, human cornea and lens are not permeable for UV light, which may result in different levels of repair activities in human retina. However, almost nothing is known about the activity of the DNA repair machinery in PR or RPE cells, be it murine or human. The post-mitotic state of neuronal retinal cells very likely hinders efficient genome editing in the retina. Together with the absence of screening and selection possibilities this is the major draw-back in *in vivo* genome editing.

In an early study, whole retina lysates were used to demonstrate DNA repair activity to correct site specific mutations in antibiotic resistant plasmids (Ciavatta et al., 2005). However, this experiment left un-addressed the differences in cell populations within the retina. The observed repair activity may have come from bipolar or ganglion cells, or even retinal glia cells. More recently, a study nicely demonstrated that especially rod photoreceptors, which represent the majority of cells in the murine retina, behave differently to DSBs compared to any other cell type in the retina. Murine rods have inverted heterochromatin organization, i.e. contain a single large chromocenter in the middle of the nucleus (Solovei et al., 2009; Solovei et al., 2013). Interestingly, Frohns and colleagues (Frohns et al., 2014) observed that adult rod photoreceptors repair only half of the induced DSBs within 1 day after damage induction, a defect that is not observed in any other cell type of the adult retina nor in rod photoreceptor precursor cells of postnatal day 4 mice. They showed that adult wild-type rods are deficient in a repair pathway involving ATM, a protein that promotes heterochromatic DSB repair by phosphorylating KAP1 and facilitating heterochromatin relaxation. Of note, they observed that rods fail to robustly accumulate active ATM at DSBs, exhibit low KAP1 levels, and display high levels of SPOC1, a factor that suppresses KAP1 phosphorylation. This leads to dramatically reduced KAP1 phosphorylation and the inability to repair heterochromatic DSBs, which together with the failure to relax heterochromatin could serve to maintain the distinct heterochromatic structure of rods. Collectively, these findings show that the unique chromatin organization of adult murine rods decrease repair efficiency for heterochromatic DSBs, providing evidence that heterochromatin

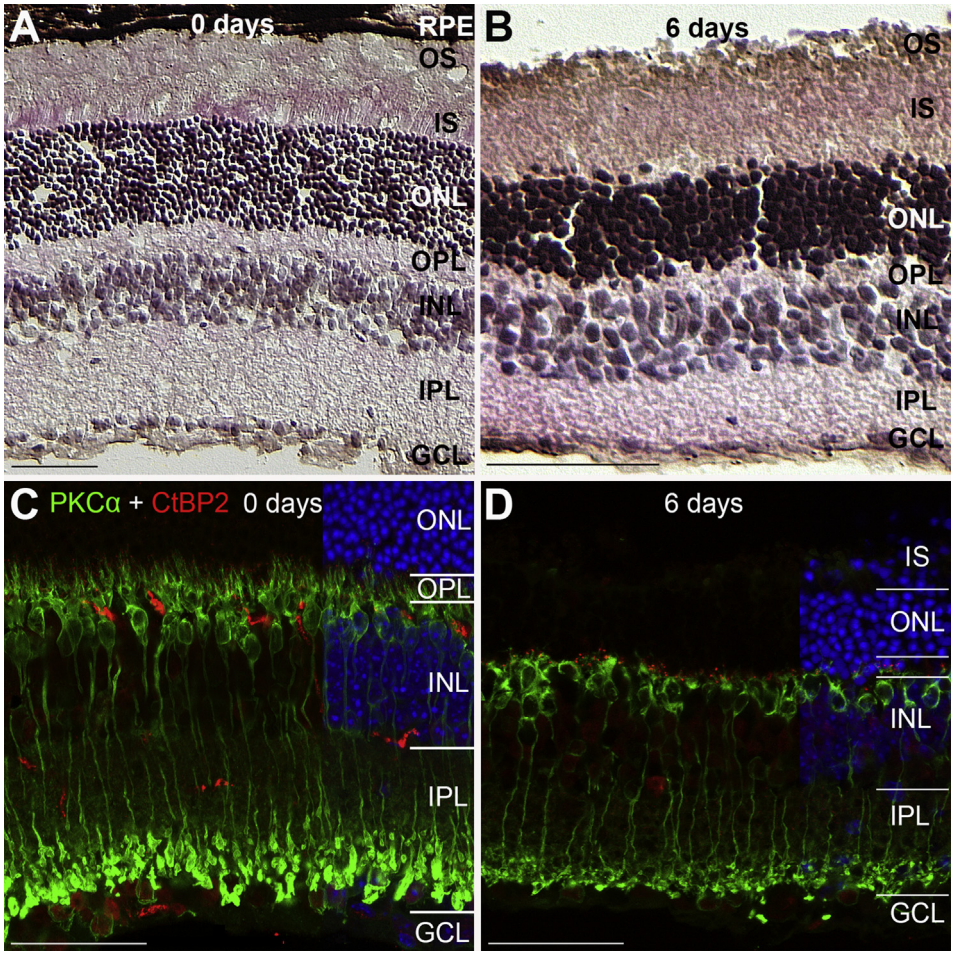

Fig. 6. Organotypic culture of adult murine retina. (**A,C**) Retina at day 0 of the culture, that is directly after euthanasia of the animal. (**B,D**) Retina at day 6 in culture. The retinae at 0 and 6 days in culture show comparable morphologic structures in hematoxilin/eosin staining (**A,B**), as well as when focussing on the photoreceptor terminals (stained with CtBP2 for the presynaptic ribbon structure) or the bipolar cells (stained with PKCa for the bipolar cells surface) (**C,D**). DAPI staining for visualizing the nuclei. ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer, Scale bar: 50 μm.

affects mammalian DSB repair *in vivo*. It remains to be seen whether rhodopsin, being the most transcribed gene in photoreceptors, might be a promising target of gene repair rather than other less transcribed loci.

Since chromatin organization in rods in humans is different from mice, it may well be that the issue of low DNA repair efficiency levels observed in mice may not be observed in humans. However, since the mouse routinely serves as model system for the development of retinal gene based therapies, scientists should consider this issue in their experimental setups.

Further work to enable precise gene correction in post-mitotic cells such as retinal neurons is critical in developing therapeutic strategies for the numerous inherited dystrophies that are currently untreatable. The solution to improve HDR in neurons will likely become evident as we improve our understanding of DNA damage repair mechanisms in neurons (Cox et al., 2015).

3.3. Neonatal versus adult retina

The adult mammalian retina as part of the central nervous system consists of terminally differentiated neurons that are in a post-mitotic state and cannot re-enter the cell cycle. Retinal glial cells (e.g., astrocytes, oligodendrocytes, and microglia) are in either a proliferative or non-proliferative state, depending on their differentiation status and possible re-entry into the cell cycle (Iyama and Wilson, 2013). The emergence of different cell types in the mouse retina occurs in two major waves (Cepko et al., 1994). The first wave peaks at E14.5, giving rise to early forming cell types, including retinal ganglion cells (RGCs), cone photoreceptors, amacrine cells, and horizontal cells. The second wave peaks at P4, giving rise to late-forming cell types, including rod photoreceptors, bipolar cells, and Müller glia cells. With eye lid opening around P13, another major change to the neuronal cell phenotype in the retina takes place, as master regulators of neuronal gene expression change the global gene expression profile within the retina (Perera et al., 2015). As a result, photoreceptors and other neurons differentiate into their final stage, having a fundamentally different gene expression profile compared to cell populations at earlier time points around birth. Hence, during the first 3 weeks after birth, a lot of terminal differentiation of retinal neurons take place, i.e. they become specialized, taking on specific structural, functional, and biochemical properties and roles. In humans, ultimately the target tissue, maturation and specialization of retinal neurons take place already in utero and is by and large finished at birth (Provis et al., 2013), even further necessitating treatment strategies and model systems that deal with differentiated neurons, like the adult mouse retina, in order to generate meaningful data prior to clinical application.

Electroporation as a method to transfer genes into the retina only works in neonatal animals (day 0) or embryonic stages,

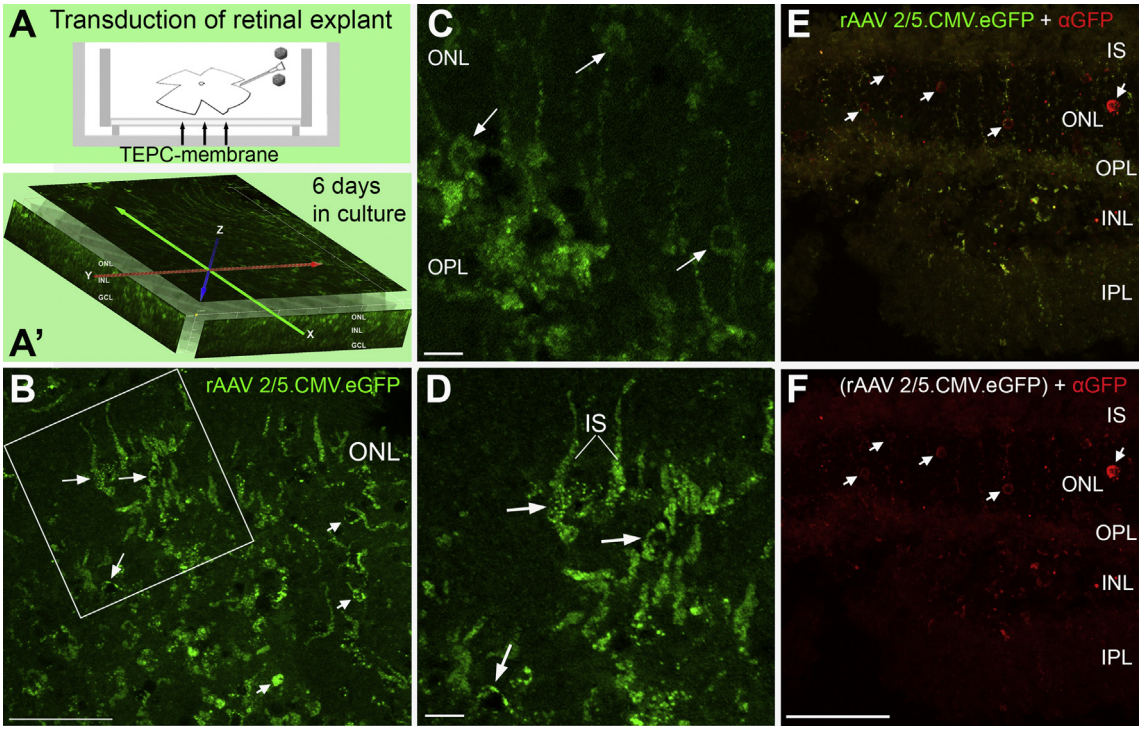

Fig. 7. Transduction of adult organotypic retina culture with AAV vectors. (**A**) The method of transduction is to add the vector between the TEPC membrane and the retina flat mount with the photoreceptors facing the membrane. After 6 days in culture, GFP signalling is visible in photoreceptors as well as some Müller cells (**B-F**). Long arrows in **B-D** indicate photoreceptor nuclei in flat mount preparation. Short arrows in **E** and **F** indicate photoreceptor nuclei in retinal sections. ONL: outer nuclear layer; OPL: outer plexiform layer; IS: inner segments; IPL: inner plexiform layer; INL: inner nuclear layer. Scale bars: 50 μm in B + F, 10 μm in C + D.

because the transfected DNA only gets in contact with the DNA molecules during cell division. Electroporation has been effective in animals for plasmid and siRNA delivery to the embryonic or neonatal retina (Matsuda and Cepko, 2004; Matsuda and Cepko, 2007; Sanuki et al., 2011). It produced lasting expression of GDNF and GFP in the retina after neonatal gene transfer (reviewed in (Bloquel et al., 2006). However, since targeted *in vivo* genome editing to repair disease causing mutations is likely to take place in fully differentiated, (i.e. mature) retinal neurons *in vivo,* gene transfer via electroporation is currently not a realistic way, and research using embryonic or neonatal animal models are not the proper experimental setting to test highly specific endonucleases and the capacity to bias the repair pathways towards HDR.

4. Preclinical approaches

Since *in vivo* approaches to treat genetic disorders have particular hurdles to overcome, information from studies not related to the retina but having been performed *in vivo* in the mouse can highlight current advancements in the field of genome editing. For example, the recent success in correcting disease causing mutations in the DMD gene encoding the dystrophin protein, causing Duchenne muscular dystrophy (DMD), shows the potentials of *in vivo* genome editing. The removal of exon 23 by *in vivo* genome editing using an AAV vector expressing CRISPR/Cas9 in a mouse model of DMD led to improved muscle function in the absence of significant off-target toxicity proofed with deep sequencing (Nelson et al., 2016; Tabebordbar et al., 2016).

Of course, cell cultures are optimal to start with when thinking about a given treatment strategy based on genome editing. One can easily design and test TALEN constructs or verify whether CRISPR/Cas9 mediated genome editing causes off target toxicity. This was done originally by several groups to test the usefulness of ZFN based genome editing approaches at the rhodopsin (Greenwald et al., 2010) or the Usher Syndrome 1 C (Overlack et al., 2012) locus. In both cases, significant activity of the designed endonucleases was observed in modified cell lines harbouring specific mutations that were addressed by the endonucleases, and off-target toxicity was marginal as verified by cell viability assays.

More recently, patient derived induced pluripotent stem cells are used to study genome editing, such as in cells derived from patients with XLRP, in which the disease causing mutation was repaired by use of the CRISPR/Cas9 system (Bassuk et al., 2016). In-depth off-target analysis was not performed so far. The idea of generating patient derived stem cells in order to correct the disease causing mutation and the use of these corrected cells to study potential treatment effects or even re-implant them into the patient's retina is the basis of the *ex vivo* genome editing approach, which has been reviewed extensively elsewhere (Wiley et al., 2015).

Working *in vivo*, the first approach often tested is to introduce organ or tissue specific changes to the genome while profiting from the advantages of dividing cells through modification of fertilized oocytes to generate founder animals that contain the altered sequence. This was done at the rd8 locus in the murine genome, which is a common defect in all mouse lines generated from the C57BL/6NJ line and which complicates phenotypic analysis of new strains because of its own impact on the mouse retina (Low et al., 2014). Single stranded oligonucleotides together with TALEN mRNA were co-injected into oocytes, resulting in up to 27% of corrected alleles in live-born animals. Frequency of Off-target toxicity events was as low as 4%, as analysed with a SNP assay to detect illegitimate recombination. A similar approach was used in rd1 mice, where the two different mutations were addressed by CRISPR/Cas9 mediated genome editing using microinjection of gRNA, single stranded oligonucleotides and Cas9 protein into

zygotes that would eventually form mosaics in the founder generation (Wu et al., 2016).

While both approaches do represent genome editing approaches in the visual system in living animals, they do not represent what we consider *in vivo* genome editing approaches (i.e. gene transfer to the retina and gene correction in situ). The first approach that does fit the definition of *in vivo* genome editing was published by Chan and colleagues in 2011, when they studied the effect of I-SceI mediated genome editing in adult ID2-hRho-GFP knock-in mice following AAV2/5 mediated gene transfer at 3–4 weeks of age (Chan et al., 2011). At 24 weeks post subretinal injection, several hundred rods were successfully repaired to express GFP (template was a duplicated segment within the target site) in a defined area of the injected zone, representing about 15% of all transduced rods within the same injected zone. Alterations at the target site were observed in almost all transduced rods, indicating that the repair machinery in adult rods is able to perform NHEJ as well as HDR with considerably high frequencies. No off-target analysis was performed in this study.

In a more recent study, a dual AAV2 vector system was used to transfer SpCas9 (first vector) and gRNA against YFP together with an mCherry expression cassette (second vector) to the retina of the Thy1-YFP mouse line via intravitreal injection with the aim to disrupt the YFP signal in the inner retina (Hung et al., 2016). Animals were between 14 and 16 weeks old at the time of the injection, and 5 weeks later, only 10% of mCherry positive cells in the inner nuclear layer were YFP positive, while 70% of mCherry positive cells were YFP positive in control animals injected with gRNA against LacZ. No off-target analysis was performed in this study.

Finally, a single subretinal injection of guide RNA (gRNA)/Cas9 plasmid in combination with electroporation at P0 generated allele-specific disruption of the murine S334ter allele in the respective S334ter-3 rat model, which prevented retinal degeneration and improved visual function at day 30 (Bakondi et al., 2016). It should be noted that the specificity of the approach cannot be transferred directly into the mouse background, because the wt mouse allele, in contrast to the wt rat allele, does contain the PAM site, which defines the specificity of the guide RNA. In addition, the developing nature of the new-born retina with dividing cells not yet differentiated into neurons, as discussed above, can only provide limited information about the effectiveness of such an approach in mature retinae. Off-target analysis was performed by Sanger sequencing of eight predicted off-target sites, and no off-target activity was shown.

5. Future directions

Deciphering the DNA repair status in the retina, and more precisely in photoreceptor and RPE cells as major target cell types is crucial before scientists can advance to efficient genome editing approaches. This knowledge will enable us to control the repair activities and potentially bias the machinery towards HDR mediated correction of the disease causing mutation. First hints that inhibition of NHEJ proteins does increase HDR activity, at least in vitro, point towards the right direction.

A second major point to be addressed represents the safe yet maximum activity of the designed endonucleases. Absence of toxicity in terms of off target activity, but also absence of immune responses to expressed transgenic proteins in retinal cells are crucial. Experience with a variety of proteins from bacterial and viral origin in retinal cells and the absence of an immune response to these proteins in primate models give positive expectations in this regard (Le Guiner et al., 2007). Genome wide toxicity assays are required to monitor off target activity of any proposed treatment approach and represent therefore critical components to the safety tool box for *in vivo* genome editing.

How about the length of the DNA strand to be corrected/replaced during the genome editing approach? Especially in mutational hot spots, such as the ORF15 exon in the RPGR gene, correction of a large part of this exon or even the entire exon would represent a single treatment approach for 80% of all patients with XLRP. But this would mean to correct almost 2000 bases in one attempt. There is currently not much known about the factors that define the length of DNA that will be corrected, the so-called DNA conversion tract length. Detailed studies employing different variants of donor template DNA is warranted to deepen our knowledge in this field.

The field of genome editing is a very active one and advances at rapid pace, potentially addressing many of these challenges in the near future,. This means that this technology may yield tremendous advantages for a large number of patients worldwide even though major hurdles for successful applications remain to be conquered.

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